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| 13. ABSTRACT (Maximum 200 words) Leishmaniasis, a disease caused by protozoan parasites of the <u>Leishmania spp.</u> , is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease are either ineffective or toxic. Malaria is considered to be the most important infectious disease of humans on a world-wide scale. It is estimated that 300 million persons are infected at any one time in the world. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis and malaria. The objective of this research was to: 1) Determine the action of the antimalarial 8-aminoquinolines on <u>Leishmania sp.</u> , 2) To identify and characterize unique DNA synthetic enzymes for the purpose of chemotherapeutic exploitation and 3) To test potential compounds sent by WRAIR and others for antiparasitic activity. | | | | |
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Table 1. The comparison between the *in vitro* test system and the *in vivo* animal test systems

| WR No. | Bottle No. | <i>in vitro</i> System (IC ₅₀ µM) | | | Animal System | | | | Conclusions on Efficiency | |
|----------------|------------|--|----------------|----------------|-------------------|------------|------------------|--------|---------------------------|------------|
| | | LM227 | T ₄ | Toxicity Index | Visceral System | | Cutaneous System | Animal | Visceral | Cutaneous |
| | | | | | <i>Leishmania</i> | Gluc Index | | | | |
| 230330 | BM16033 | 0.04 | 10 | 0.004 | active | 632 | - | - | similar | - |
| 230330 | BH32724 | 2 | 100 | 0.02 | none | - | - | - | dissimilar | - |
| 247733 | BJ52043 | 40 | 5 | 8 | none | - | - | - | similar | - |
| 180409 | BE99420 | 40 | 2 | 20 | none | - | none | toxic | similar | similar |
| 247734 | BJ52052 | 50 | 10 | 5 | none | - | - | - | similar | - |
| 006026 | BK01845 | 50 | 80 | 0.63 | active | 594 | active | toxic | similar | similar |
| 268317 | BL59588 | 70 | 460 | 0.15 | none | - | - | - | similar | - |
| 074086 | AH95665 | 170 | 120 | 1.4 | none | - | - | - | similar | - |
| 243251 | BL21100 | 178000 | 92 | 1935 | none | - | - | - | similar | - |
| 260547 | BL34170 | >> 150 | 26 | >> 5.8 | none | - | - | - | similar | - |
| 182393 | BL56390 | >> 292 | 96 | >> 3.0 | none | - | - | - | similar | - |
| 004251 | BK97823 | 100ng/ml | none | > 1 | none | - | - | - | dissimilar | - |
| 053188 | ZB27990 | none | none | - | none | - | - | - | similar | - |
| 042934 | ZB27758 | none | 6 | < 1 | none | - | - | - | dissimilar | - |
| 030091 | ZB27641 | none | < 100 | > 1 | none | - | - | - | similar | - |
| 256363 | BL29446 | none | 1 | > 1 | none | - | - | - | dissimilar | - |
| 255706 | BL20934 | none | none | - | none | - | - | - | similar | - |
| 030090 | BE16494 | none | 70 | > 1 | none | - | - | - | similar | - |
| 029252 | AJ32190 | none | 100 | > 1 | none | - | - | - | similar | - |
| 098657 | AF55410 | none | none | - | none | - | - | - | similar | - |
| 229870 | BL06916 | none | none | - | active | 0.75 | active | none | dissimilar | dissimilar |
| 254847 | BL58705 | 0.21 | - | - | active | 208 | - | - | similar | - |
| 182971 | ZP64868 | - | 1004 | - | none | - | active | none | similar | - |
| Predictability | | | | | | | | | 78% | 67% |

Note: LM227 - *Leishmania mexicana* 227; T₄ - human lymphocytes CEM T₄; none - no inhibition or no toxicity;
 Toxicity Index = IC₅₀ to LM227 / IC₅₀ to T₄; Gluc Index - Glucantime Index.

COMPARISON OF THE *in vivo* AND *in vitro* TEST SYSTEMS

According to the results shown in Table 1, the *in vitro* system developed in our laboratory predicated the results of the animal test systems fairly well. The results indicated a 78% predictability for the visceral and 67% for the cutaneous leishmaniasis. These comparisons take into account the antileishmanial activity of the test compounds as well as their toxicity.

Our test system used *L. mexicana* 227 promastigotes (1 and 2). Therefore, the *in vitro* system gave a higher accuracy on the prediction of the visceral leishmaniasis system than that of the animal cutaneous leishmaniasis system. Various scenarios might account for differences between the *in vitro* and *in vivo* system. In the animal test systems (3 ~6), the tested compounds might have been digested by the animal's metabolism to an active or inactive metabolite. The leishmanial cells in the *in vitro* system were in the promastigote form as opposed to the amastigote form in the animal test system.

Conditions in the animal test system are more similar to the human body than that of the *in vitro* cellular test system. But the *in vitro* system has many advantages as a preliminary drug screen: 1) Because cells are directly exposed to the tested compounds in the *in vitro* system, this system can reveal the direct effects of the compounds without the confusion of metabolism of the test compounds; 2) it is time efficient. The *in vitro* test system takes from 48 to 72 hours to be accomplished; 3) it is more economical; 4) the *in vitro* system reduces the need for animals and is in compliance with the animal right act.

Our *in vitro* test system overpredicts toxicity but more testing with various cell lines will increase our toxicity predictability. We are in the process of developing a chicken embryo *in vitro* cell system to use in conjunction with our cell toxicity studies.

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THE MODE OF ACTION OF 8-AMINOQUINOLINE IN *Leishmania mexicana*

I. OBJECTIVE AND RATIONALE

Leishmania species in the family of trypanosomatid protozoa share many common biochemical characteristics with malarial organisms. Both of them are protozoa, transmitted by insects and are intracellular parasites. Therefore some of the antimalarial drugs are being screened for their antileishmanial activities. 8-aminoquinoline compounds developed for their antimalarial activity were tested in *Leishmania mexicana* to determine their inhibitory properties.

Three antimalarial 8-aminoquinoline compounds (WR006026, WR238605 and WR242511) demonstrated strong inhibition to the growth of *Leishmania* in a microwell plate test. Their IC_{50} s (concentration at 50% inhibition) were 50 μ M, 4.0 μ M and 2.5 μ M respectively against *Leishmania mexicana* 227. WR242511 was chosen because it exhibited the most antileishmanial activity. The mode of action of WR242511 on DNA, RNA, protein and fatty acid synthesis of *Leishmania* was investigated utilizing radioactive substrates. The results suggest that 8-aminoquinoline inhibits DNA synthesis initially, then, the rate of RNA synthesis declines. With time protein synthesis is the last metabolic pathway to be affected. This is possibly due to the inhibition of DNA and RNA synthesis. The inhibition of fatty acid synthesis is observed after the inhibition of RNA synthesis and 2 hours after incubation with WR242511.

It has been reported that 8-aminoquinolines are inhibitory to bacteria. For this reason we investigated the effect of WR242511 on the kinetoplast DNA of *Leishmania mexicana*. Type II topoisomerase controls the catenation and supercoiling of minicircle DNA of the kinetoplast DNA (kDNA) network in mitochondria. Type II topoisomerase in mitochondria of *Leishmania mexicana* 227 was studied by detecting the decatenation of the minicircles from the kDNA network. The results demonstrated that the free minicircle DNA increased in *L. Mexicana* 227 cells when they were treated with WR242511. The results suggest that WR242511 interferes with the catenation and decatenation of mitochondria kDNA network (which composes of 20% of total leishmanial DNA) through inhibition of the type II topoisomerase. Subsequently inhibition of DNA synthesis leads to the death of the leishmanial cells.

II. INTRODUCTION

Leishmania sps. are in the family of trypanosomatid protozoa. Leishmaniasis is endemic in 80 countries with 350 million people at risk, 12 million infected and an annual incidence of 3-4 million. Usually it is transmitted by the blood-sucking phlebotomine sandfly. *Leishmania* exists in two forms: extracellular flagellated form (promastigote) and intracellular non-motile form (amastigote). There have been limited biochemical investigations of *Leishmania* species susceptibility to aminoquinolines.

Leishmanial protozoa shares many common biochemical characteristics with malarial organisms. Both of them are protozoa, transmitted by insects and intracellular parasites.

Therefore some of the antimalarial drugs are being screened for their antileishmanial activities. We are in the process of testing antimalarial compounds for antileishmanial activity and performing mode of action studies.

1. 8-Aminoquinoline Compounds

The antimalarial and antiseptic activity of 8-aminoquinoline compounds were first found by German scientists (7). They were developed as a substitute for quinine. Chloroquine, a 4-aminoquinoline compound, has been the most used antimalarial drug for about 40 years. Today, many malarial strains demonstrate resistance to chloroquine (8) and other antimalarial drugs. In the past 20 years, more and more U.S. civilians have been infected by malaria. Recently, to study the problem of drug-resistance the mode of action of 8-aminoquinoline compounds is being investigated. These drugs have strong activity against both the wild types and some multi-drug-resistant strains. Primaquine is an approved and widely used agent for the antimalarial treatment (9 and 10). Many other 8-aminoquinoline compounds are being developed by Walter Reed Army Institute of Research (WRAIR), such as WR242511, WR238605 and WR006026, which have been shown to be very effective for the treatment of leishmaniases (11), malaria (9 and 12), and pneumonia (13, 14 and 15). WR242511 (16) has shown the best activity as an antileishmanial compound. Its mode of action and toxicity to host cells is currently being investigated in our laboratory.

2. The Mode of Action of Quinolines

So far, four hypotheses have been proposed for the mode of action of these quinoline-containing antimalarial compounds such as the chloroquine (a 4-aminoquinoline compound):

1) DNA Intercalation. The evidence for intercalation suggests that these compounds can

specifically bind to poly(dG-dC) DNA sequence at low salt concentrations. It is suggested that these compounds might be toxic to the parasite by selectively accumulating in specific genes and inhibiting their expression (17); 2) Lysosome membranes. These compounds can diffuse across lysosome membranes. They are protonated and accumulated in lysosome of the malarial parasite but not in the human blood cells (18); 3) Ferriprotoporphyrin IX (FPIX). Parasites digest human hemoglobin to obtain nutrition and release large amounts of the toxic FPIX. The quinoline-containing compounds can bind with FPIX to accumulate in the parasitic acid food vacuole leading to starvation of the parasite (19); 4) Inhibition of heme polymerase. Quinoline-containing compounds can inhibit heme polymerase that polymerizes and detoxifies FPIX (20 and 21).

3. Quinolone Compounds and Type II Topoisomerase in Mitochondria

Type II topoisomerase is an enzyme which cleaves both strands of the double helix DNA simultaneously and passes another double-helical segment through this break. This function allows the DNA to be winded and unwinded, supercoiled and relaxed which is very important during DNA replication.

Mitochondrial DNA is a double stranded closed circular molecule and negatively supercoiled and a means is required for introducing such superhelical turns. Kinetoplast (network) DNA (kDNA) in protozoan mitochondria consists of thousands of 1 kb minicircles and a few 20 kb maxicircles (22). The minicircles have been shown to be decatenated from the network prior to their replication and then rejoined to the network (23). The presence of these large highly catenated DNA networks within the single mitochondrion of trypanosomes

reflects the presence of an abundant type II topoisomerase in the mitochondrion of these organisms.

Type II topoisomerase has been found in mitochondria of many different cells such as *Plasmodium berghei* (24, 25 and 26), *Leishmania* (27) and mammalian cells, and it has been purified by utilization of novobiocin affinity chromatography from protozoa (*Crithidia fasciculata*) mitochondria (28). Immunoblots show at least a 10-fold higher level of topoisomerase II in preparations of partially purified mitochondria as compared with those from whole cells of *Crithidia fasciculata*.

Analogues of quinolone are widely used antimicrobial agents which were originally developed during antimalarial research (29 and 30), and have similar molecular structures to those of 8-aminoquinoline compounds. It has been found that quinolones inhibit the activity of type II topoisomerase, which can decatenate and relax the supercoiled DNA in both bacteria (31 and 32), protozoa *Crithidia fasciculata* mitochondria (28) and many other microbes (31, 32 and 33).

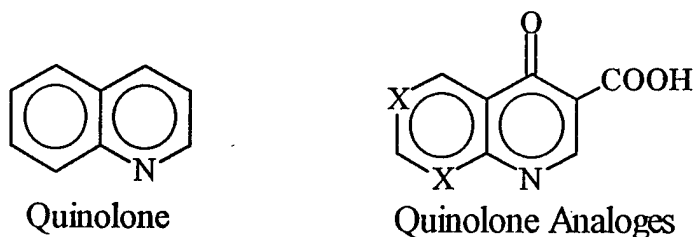


Figure 1. The structure of quinolone and its analogs

4. The Mode of Action of 8-Aminoquinoline Compounds

8-aminoquinoline compounds, such as WR242511 (16) were synthesized during antimalarial studies. Some 8-aminoquinolines have demonstrated strong inhibition to the growth of the leishmanial parasites. WR242511 can cause dilatation of the nuclear envelopes

and membranous arrays arising from the reticular system in *Pneumocystis carinii* initially, and latter the large arrays of smooth membranous materiel (13). However the exact mode of action of 8-aminoquinoline compounds in both *Plasmodium falciparum* and *Leishmania mexicana* cells remains speculative.

Quinolone and 8-aminoquinoline compounds are very similar in their molecular structures. Therefore, it is conceivable that they might share a similar mode of action. Because the minicircles of DNA in the kinetoplast DNA network are decatenated from network prior to their replication and then rejoined to the network, the activity of type II topoisomerase can directly affect the catenation and decatenation of minicircle DNA.

In this research, WR242511 (Bottle No. BM05816, Figure 2) obtained from Walter Reed Army Institute of Research, was investigated for its antileishmanial activity. Also the toxicity of this compound was determined using human CEM T₄ cells in an *in vitro* assay developed in our laboratory (34). By utilizing radioactive substrates in an incorporation assay, the effects of WR242511 on DNA, RNA, protein and fatty acid synthesis were studied to determine the mode of action of the compound. Another object of our researches was to determine the effect of WR242511 on the amount of free minicircles in treated leishmanial cells compared with untreated control cells, by using a radioactive minicircle DNA probe.

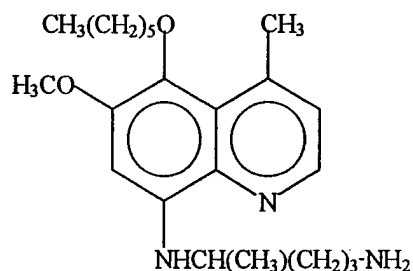


Figure 2. Structure of WR24251

III. MATERIALS AND METHODS

1. Chemicals

8-Aminoquinoline Compound and Radioactive Substrates. WR242511 (Figure 2), WR238605 and WR006026, were obtained from Walter Reed Army Institute of Research. The radioactive substrates of DNA, RNA, protein and fatty acid synthesis were [2-¹⁴C]thymidine, [2-¹⁴C]uridine, [1-¹⁴C]L-leucine and [1-¹⁴C]acetic acid respectively (Moravsek Biochemicals). The radioactive minicircle DNA probe was prepared from 3000 Ci/mMol ³²P-dCTP (New England Nuclear).

2. Cell Lines and Medium

The leishmanial species used in these studies was *Leishmania mexicana* (Walt Reed stain 227). It was maintained and tested in Steiger and Black media (35). For the investigations of the mode of action of WR242511, Steiger and Black media containing 0.23 mM L-leucine was used in the incorporation assay. To determine possible mammalian cell toxicity, human lymphocytes CEM T₄ cells grown in RPMI 1640 media (Gibco BRL) supplemented with 5% fetal calf serum were studied.

3. Inhibition and Toxicity Test

The inhibition of WR006026, WR238605 and WR242511 on the growth of *Leishmania mexicana* 227 was tested in a microwell plate assay (34). It was observed by comparing the cell turbidity of *L. mexicana* treated with WR242511 to that of control cells at 660 nm at 25°C within 48 hours. The toxicity of WR242511 on Human lymphocyte CEM T₄ cells was tested in a test tube assay incubating with 5% CO₂ at 37°C for 48 hours.

4. Incorporation Test on DNA, RNA and Protein Synthesis

The *L. mexicana* 227 cells were incubated in Steiger and Black media (with 0.23 mM leucine) with 5.0 μ M WR242511. The radioactive substrates, 72 nmol (0.4 μ Ci) of [14 C]thymidine, 18 nmol (0.1 μ Ci) of [14 C]uridine and 144 nmol (0.4 μ Ci) of [14 C]L-leucine, were added to 20 ml of *L. mexicana* cell culture (1×10^6 cells/ml) to study the incorporation of DNA, RNA and protein synthesis respectively. At 0, 0.5, 1, 2, 4, 6 and 8 hour after the radioactive substrates had been added, 1 ml sample was taken from each test and was mixed with 9 ml of ice cold 10% trichloroacetic acid. Samples were filtered through G4 glass fiber filter circles (Fisher) using a Manifold (Waters), and washed twice with cold 5% trichloroacetic acid and ethanol. The radioactivity on the dried filter circles was counted with a Delta 300 liquid scintillation counter (Searle Analytic Inc.). The cell cultures were maintained at 25°C. During the experiment, the cell densities were examined at 2 hour intervals.

5. Fatty Acid Synthesis Assay

The incorporation of radioactive substrate for fatty acid synthesis was tested with the *L. mexicana* 227 cells in Steiger and Black media (with 0.23 mM leucine). 36 nmol (2 μ Ci) of [14 C]acetic acid was added in 10 ml *L. mexicana* cell culture treated with 5.0 μ M WR242511. At 0, 0.5, 1, 2, 4, 6 and 8 hour, the fatty acids were extracted by a methanol-chloroform mixture as described by Ames (36 and 37). 320 μ l samples were taken from the culture and added to 600 methanol/chloroform (2:1 v/v). The mixtures were diluted with 920 μ l water and 920 μ l chloroform. 800 μ l of fatty acid phase (bottom layer) was taken and its radioactivity was counted by Delta 300 liquid scintillation counter.

6. Free Minicircle DNA Assay

A. ^{32}P -minicircle DNA probe

1) Preparation of *L. mexicana* 227 Crude Mitochondria (38)

L. mexicana 227 cells in 1 L Brain Heart Infusion media were harvested by 8000xg 10 min centrifugation and washed twice with 50 ml of 0.05 M Tris-HCl (pH 7.4). The cells were resuspended at 0°C in 50 ml of digitonin lysis buffer (DLB; 0.25 M mannitol, 10 mM MOPS, pH 7.5, 250 μM MgCl_2 , 250 μM EDTA, 5 μM L-ascorbic acid, 0.6 mg/ml polyvinylpyrrolidone, 0.3 mg/ml BSA, 0.2 mM PMSF, and 1 $\mu\text{g/ml}$ leupeptin). The cells were treated with 18 ml digitonin solution (0.1 g of digitonin/ml of *N,N*-dimethylformamide, diluted 1:10 in DLB). After swelling on ice for 2 hour, the cells were centrifuged (5000 x g, 10 min, 4°C) and resuspended in 50 ml of fresh DLB without digitonin. The cells were then lysed with a Polytron (Brinkmann Instruments) at 4°C. The lysate was centrifuged (27,000xg, 30 min at 4°C), and the pellet were resuspended in 50 ml of STE (250 mM sucrose, 20 mM Tris-HCl, pH 7.9, 20 mM EDTA). The crude mitochondria was collected by centrifugation (27,000xg, 30 min, 4°C).

2) Determination of the Minicircle DNA

kDNA isolation: 2 g of crude mitochondria were mixed with 2 ml PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.7 mM KH_2PO_4 , pH 7.4) and 3 ml lysis buffer (0.5 M EDTA, pH 9.0, 1% N-lauroylsarcosine and 1 mg/ml proteinase K). After incubation for 24 hours at 55°C, the lysate was diluted with 5 ml TE (10mM Tris-HCl, pH 7.5, and 1 mM EDTA) buffer. The DNA was purified by phenol/chloroform method, precipitated by isopropanol and washed with 70% ethanol twice. The DNA was desolved in 3 ml TE buffer and was used for the isolation of kDNA and for the purification of Minicircle DNA. DNA solution

(1.5 ml) was loaded onto 10 ml of 20% sucrose NET100 cushion (100mM NaCl, 100 mM EDTA, and 10 mM Tris-HCl, pH 8.0), and centrifuged at 40,000xg for 1 hour. The kDNA along with some other large DNA was collected from the bottom of the centrifuge tube, and dialyzed against TE buffer.

Restriction enzyme digestion: 40 µl of the DNA above was transferred into six of 1.5 ml microcentrifuge tubes. Each tube contained 2 µl of the following restriction enzyme: Kpn I, Hinc II, Hind III, Xho I, BamH I or EcorR I. The tubes were incubated at 37°C in a water bath for 2 hours. The digested DNA was loaded with blue loading buffer onto a 1.5% agarose gel (140 V for 2 hours). 1 kb ladder was used as DNA size maker. The size of minicircle DNA was determined from this procedure.

3) Purification of Minicircle DNA

Crude mitochondria (0.2 g) were lysed as previously described. The lysate was mixed with 160 µl of gel loading buffer (with blue dye), and then loaded with DNA marker onto 1% agarose gel with 0.8 µg/ml ethidium bromide. The gel was run at 140 V and 40 mA (0.5 seconds forward and 0.25 seconds reverse) for 6 hours at room temperature (PC750 Pulse Controller, Hoefer Scientific Instruments). The DNA band corresponding to minicircle was cut off from the gel, and placed into a 1.5 ml microcentrifuge tube with 100 µl TE. The gel was ground with a plastic homogenizer. An equal volume of buffer saturated phenol was added and vortexed until mixed well, and the sample was placed at -70°C for 1 hour. It was thawed at 37°C and then spun for 5 min in a Fisher Micro-centrifuge (Model 235 A). The supernatant was transferred to a new tube. An equal volume of phenol/chloroform was added and vortexed. The mixture was spun for 5 min at Fish Micro-centrifuge. The supernatant was

transferred to a new tube and an equal volume of chloroform was added, vortexed, and spun for 5 min. The supernatant was transferred to a new tube. 1/10 volume of 3M NaAC (pH 6.8) and an equal volume of iso-propanol was added. The tube was placed in -70°C for 15 min, and thawed. The DNA was centrifuged for 15 min. The DNA pellet was washed twice with 70% ethanol, dried and resuspended in 50 µl of TE.

4) Labeling of minicircle DNA with ^{32}P -dCTP.

Purified minicircle DNA was transferred into a 1.5 ml heat tolerant tube, and denatured in boiling water for 5 min. 8 µl of 250 µCi/25 µl ^{32}P -dCTP and 18 µl LS buffer containing dATP, dTTP and dGTP was added into the denatured DNA. 1 µl of Klenow DNA polymerase (5U/µl) was added and placed at room temperature for 12 hours to label the minicircle DNA. The labeled probe was centrifuged through a 1 ml of G-50 column to eliminate the free radioactive reagent and stored at -20°C.

B. Preparation of *L. mexicana* 227 cells

200 ml BHI media was inoculated with 30 ml *L. mexicana* 227 cell culture (O.D. = 0.4 at 660 nm in test tube). After 24 hours incubation at 25°C, the cells were in log phase and were used in these experimental studies.

1) *L. mexicana* 227 were exposed to WR242511 at the varying concentrations. The O.D. reading at 660 nm was around 0.4 in a 5 ml test tube. 20 ml of *L. mexicana* 227 cell culture was added into each 25 cm² cell culture flask (Corning). 50 µl of WR242511 in DMSO at varying concentrations was added to the cell cultures making the final concentrations of 1.25, 2.5, 5.0, 10, 20 and 30 µM respectively. The control contained 50 µl of DMSO. After 8 hours of incubation at 25°C, the cells were lysed.

2) Effect of WR242511 with time. 20 ml of a *L. mexicana* 227 log phase cell culture was placed in each 25 cm² cell culture flask (Corning). At 0, 15, 30 min and 1, 1.5, 2, 4 and 6 hours, 50 µl of WR242511 at different concentrations was added to the cells to make the final concentration to 20 µM. The control had 50 µl of DMSO. After 8 hours of incubation at 25°C, the cells are ready for lysis.

C. Cell Lysis

After incubation at various times and concentrations with WR242511, *L. mexicana* 227 cells were centrifuged at 8000 xg 10 min, and washed with 10 ml of 0.05 M Tris-HCl (pH 7.4). The cell pellets were resuspended in the same Tris-HCl buffer and transferred into test tubes. By the addition of Tris-HCl buffer, the cell densities were adjusted to the same O.D. reading at 660 nm. Samples of 1 ml were transferred to 1.5 ml microcentrifuge tubes and spun in a Fisher Micro-centrifuge. The supernatant was removed and the cell pellets were saved for lysis. The cells were lysed in 50 µl PBS and 30 µl lysis buffer in a microcentrifuge tube at 55°C for 24 hours.

D. Southern Blot

1) Pulsed field Electrophoresis:

70 µl of each lysate was mixed with 7 µl of 10 x blue gel loading buffer. 70 µl of each mixture was loaded onto 1% agarose gel with 0.8 µg/ml ethidium bromide. The gel was run at 140 V and 40 mA (0.5 seconds forward and 0.25 seconds reverse) for 6 hours at room temperature.

2) DNA Transfer:

Using the capillary transfer method, DNA fragments were carried from the gel in a flow of liquid and deposited on the surface of the Nylon Membranes (Figure 3. Molecular Cloning, 2nd Edition). In this experiment, DNA was transferred to nylon membranes under alkaline conditions as described (Molecular Cloning, 2nd Edition): After electrophoresis, the agarose gel was transferred onto a glass plate and any unused areas of the gel were trimmed away with a razor blade. This DNA was denatured by soaking the gel for 15 min in a denaturation solution (0.4 N NaOH and 1 M NaCl) with constant shaking. The solution was changed and gel was soaked for another 20 min. While the gel was soaking in the denaturation solution, a piece of nylon membrane (NYTRAN, Schleicher & Schuell) was cut about 1 mm larger than the gel in both dimension. The membrane was floated on the surface of a dish of deionized water until it was completely wet from beneath, and then the membrane was immersed in denaturation solution for at least 5 min. A corner was cut from the membrane as a mark of orientation. The denatured DNA was transferred from the gel to the membrane by capillary action as described in Figure 3. The gel was peeled from the membrane and discarded. The membrane was neutralized the membrane and cleaned by soaking in 0.5 M Tris-HCl (pH 7.2) and 1 M NaCl for 15 min at room temperature. The membrane was removed from the neutralizing solution and placed on a paper towel to dry for at least 30 min at room temperature. The dried membrane was placed between two pieces of 3MM paper (Whatman), and baked for 2 hours at 80°C in a vacuum to fix the DNA to the membrane.

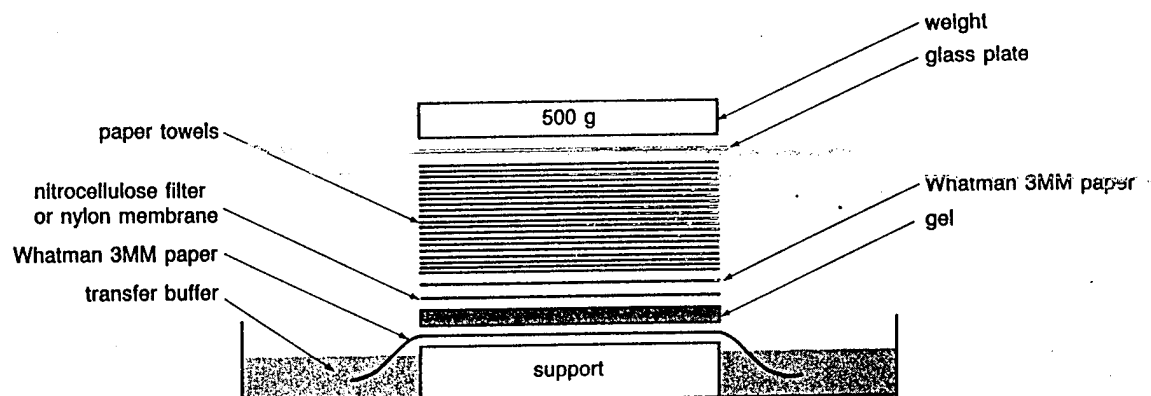


Figure 3. Capillary transfer of DNA from agarose gels. 10 x SSC buffer was drawn from a reservoir and passed through the gel into a stack of paper towels. The DNA was eluted from the gel by the moving stream of buffer and was deposited on a nylon membrane. A weight applied to the top of the paper towels helped to ensure a tight connection between the layers of material used in the transfer system.

E. Hybridization of ^{32}P -minicircle DNA probes to immobilized DNA and Autoradiography

The nylon membrane containing the transferred DNA was floated on 6x SSC solution and then transferred it into 30 ml of hybridization solution (6 x SSC, 5 x Denhardt's reagent, 0.5% SDS 100 $\mu\text{g}/\text{ml}$ denatured, fragmented salmon sperm DNA and 50% formamide) in a tray and incubated at 42°C overnight. The hybridization solution was poured out, and then the nylon membrane was washed three times with 200 ml of 0.5 x SSC and 0.05% SDS at 65°C for 30 min.

The membrane was placed on a sheet of 3MM Whatman filter, and marked with radioactive ink to align the autoradiograph with the filter. Then it was wrapped with plastic wrap. The membrane was exposed to X-ray film (Fuji RX film) at -80°C overnight to obtain an autoradiographic image.

IV. RESULTS

1. Inhibition Assay

WR242511 demonstrated strong inhibition against *Leishmania mexicana* while it also inhibited the growth of human lymphocyte CEM T₄ cells. Its MIC₅₀ (concentration at 50% inhibition of the cell growth) to *L. mexicana* and T₄ cells were 2.5 μ M and 5.0 μ M respectively (Figure 4).

2. Incorporation Assay

The incorporation assay (Figure 5) demonstrates that the inhibition of [¹⁴C]leucine incorporation was negligent in the first 2 hours. It increased with the time and the maximum inhibition obtained was only 30% at the end of 8 hours. There was little inhibition of incorporation of [¹⁴C]acetic acid into fatty acids within the first hour. But it sharply increased to 50% after 2 hours. The maximum inhibition was 64% at 4 hour. The incorporation of [¹⁴C]thymidine and [¹⁴C]uridine was strongly inhibited (70 ~ 90%) by 5.0 μ M WR242511. During the first 4 hours, the inhibition of [¹⁴C]thymidine incorporation (91%) was greater than that of [¹⁴C]uridine incorporation (79%). But at the end of 8 hours, WR242511 exhibited stronger inhibition on [¹⁴C]uridine incorporation. These results suggest that DNA synthesis maybe the target of inhibition by WR242511 in *L. mexicana* (Figure 5).

3. Free Minicircle DNA Test

A. Minicircle DNA and ³²P-minicircle DNA probe.

If a restriction enzyme can cut the minicircle at more than one locations, the products of digestion will be more than one small band, and these products digested by different enzymes should have different sizes. But in our experiment, Xho I, EcoR I, Hind III and BamHI cut the network DNA and produced the same 2.7 kb DNA band (Figure 6). This suggests that these enzymes must have linearized the minicircle DNA by giving it a single cut. The minicircle DNA of *L. mexicana* 227 was determined to be about 2.7 kb.

The southern blot analysis demonstrated that the radioactive probe made from the 2.7 kb DNA band could only bind to the 2.7 kb band and the network DNA which could not move out from the well on the gel (Figure 7). This confirmed that the 2.7 kb DNA is the minicircle DNA released from kDNA networks digested by restriction enzyme. Xho I was the most effective enzyme in the cleavage of minicircle from kDNA network.

B. WR242511 treatment at different concentrations.

According to the autoradiographic film, the amount of free minicircle DNA increases when leishmanial cells are exposed to WR242511 at 10 μ M or higher. At 20 and 30 μ M, WR242511 significantly increased the free minicircle and decreased the catenated minicircle DNA in leishmanial cells.

C. Treatment of *L. mexicana* 227 with WR242511 at varying times.

When cells were treated with 20 μ M WR242511 for 2 to 8 hours, the amount of free minicircle DNA increased and the minicircle DNA in networks decreased (Figure 9). WR242511 significantly affected the minicircle release and catenation within the first 2 hours (Figure 10). Although the increase of the free minicircle DNA in 20 μ M of WR242511 treated *L. Mexicana* 227 could be found in the first 15 minutes after exposure of cells to the drug.

Figure 4. IC₅₀ of 8-Aminoquinolines to *L. mexicana* 227 and Human T4 cells

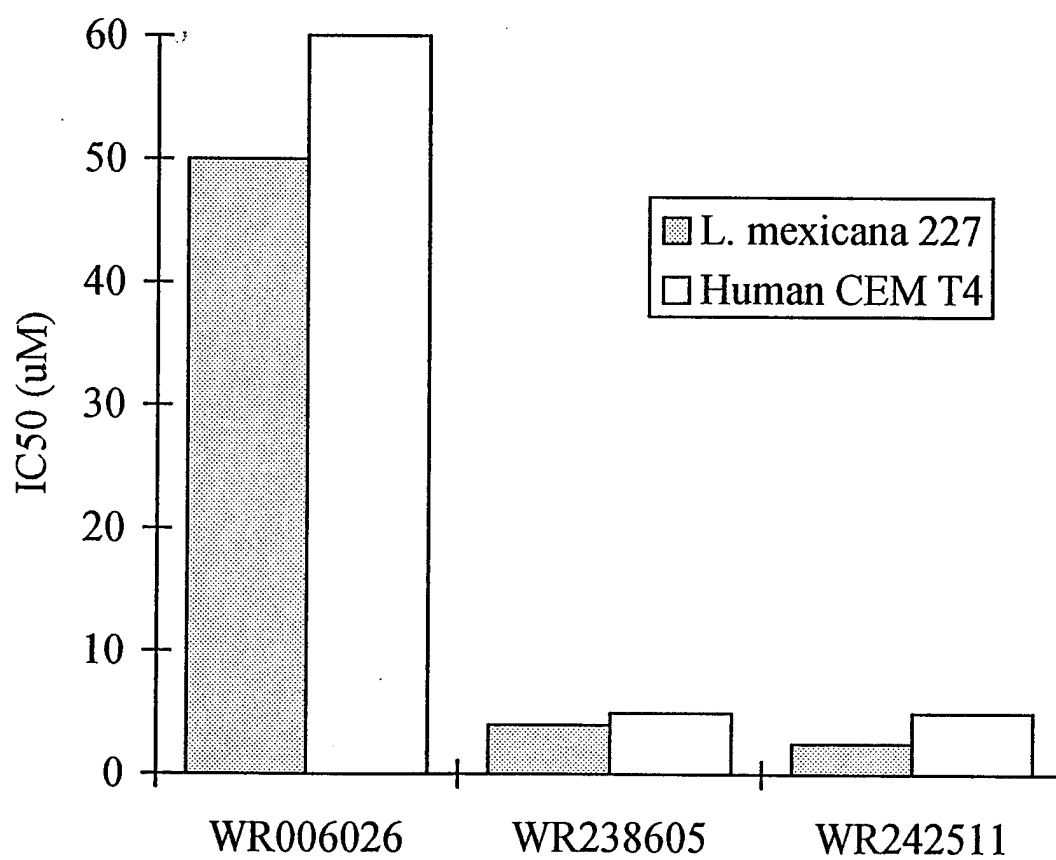
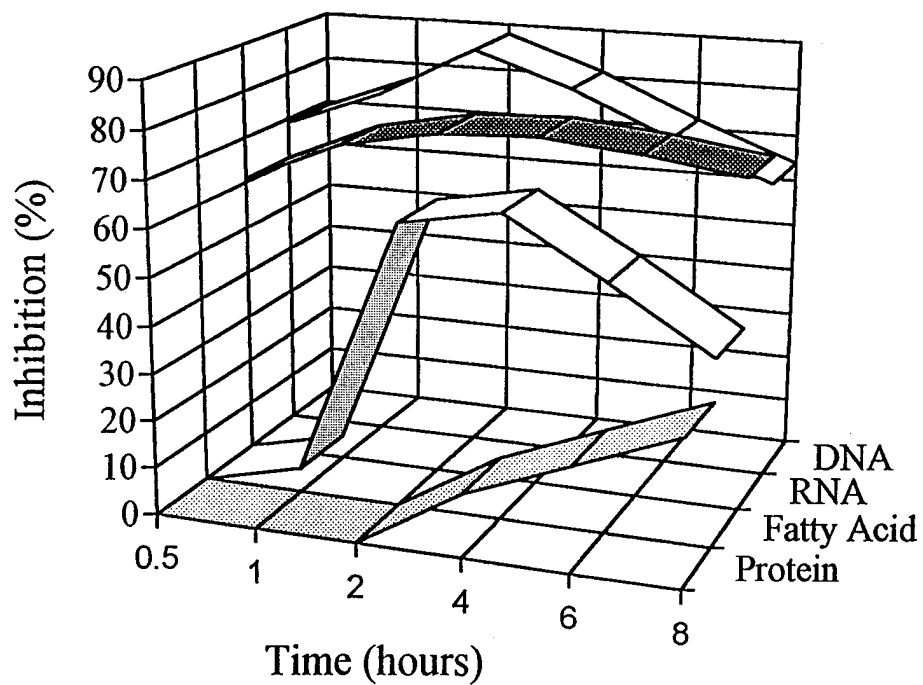


Figure 5. The Inhibition of Metabolic pathway of *L. mexicana* by WR242511



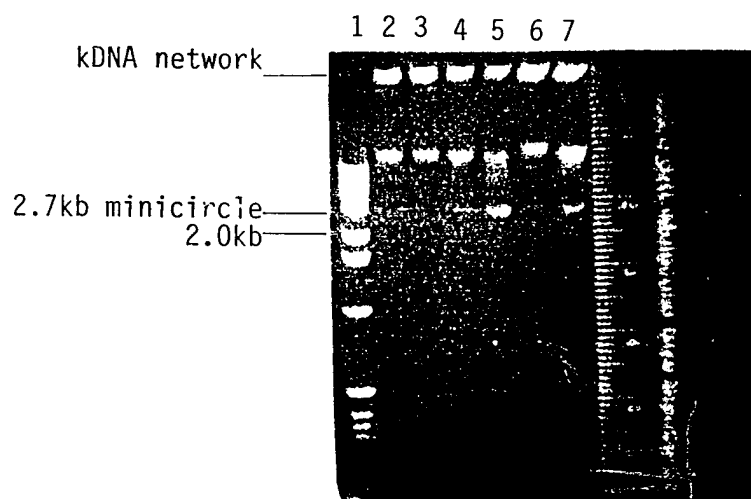


Figure 6. The electrophoresis of the DNA digested by restriction enzymes. Lane 1 is 1 kb DNA ladder; Lane 2, 3, 4, 5, 6 and 7 were the DNA digested by Kpn I, Hinc II, Hind III, Xho I, BamH I and EcorR I respectively. The kDNA networks were too large to move out of wells. The 2.7 kb bands were the minicircle DNA.

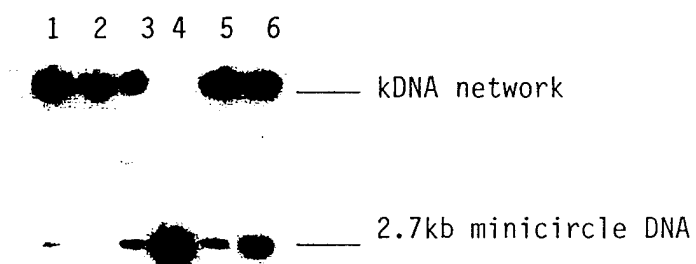


Figure 7. The southern blot of the minicircle DNA.
 Lane 1, 2, 3, 4, 5 and 6 were the DNA digested by Kpn I, Hinc II, Hind III, Xho I, BamH I and EcorR I respectively. The kDNA networks were too large to move out of wells. The 2.7 kb bands were the minicircle DNA. The radioactive probe could only bind to minicircle and network DNA.

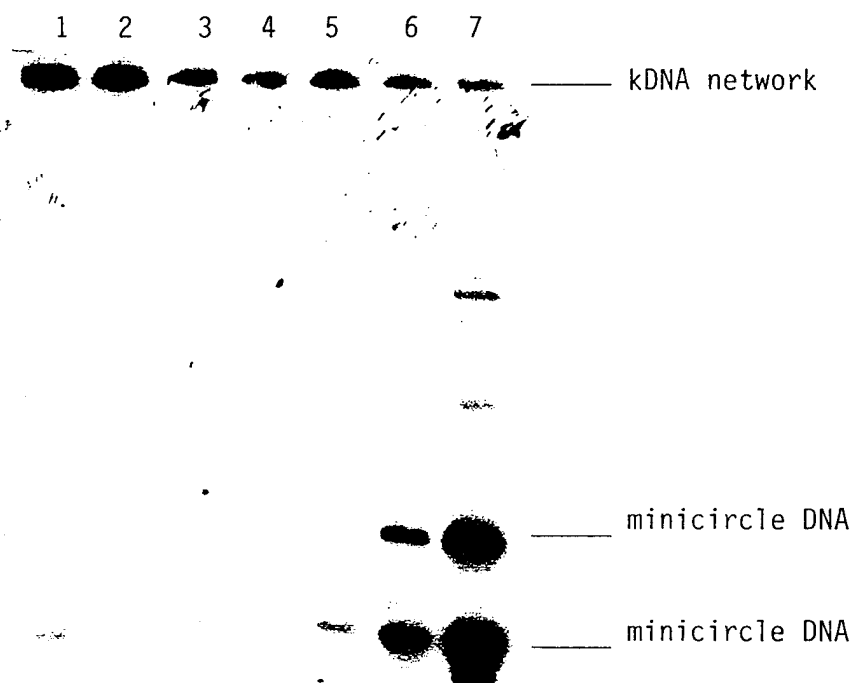


Figure 8. Southern blot of WR242511 at different concentrations. Lane #1, 2, 3, 4, 5, 6 and 7 corresponds to WR242511 treatment at 0, 1.25, 2.5, 5.0, 10, 20 and 30 μ M

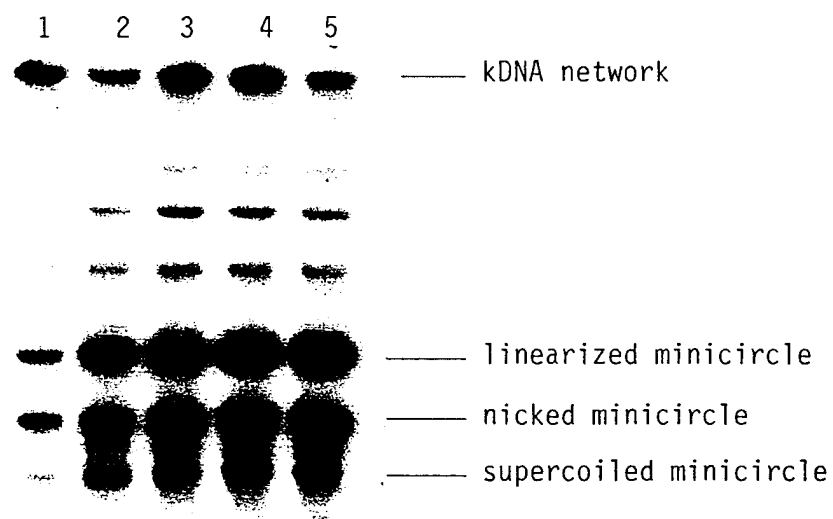


Figure 9. Southern blot of WR242511 treatment for varying times. Lane #1, 2, 3, 4 and 5 correspond to 20 uM WR242511 for 0, 2, 4, 6 and 8 hours.

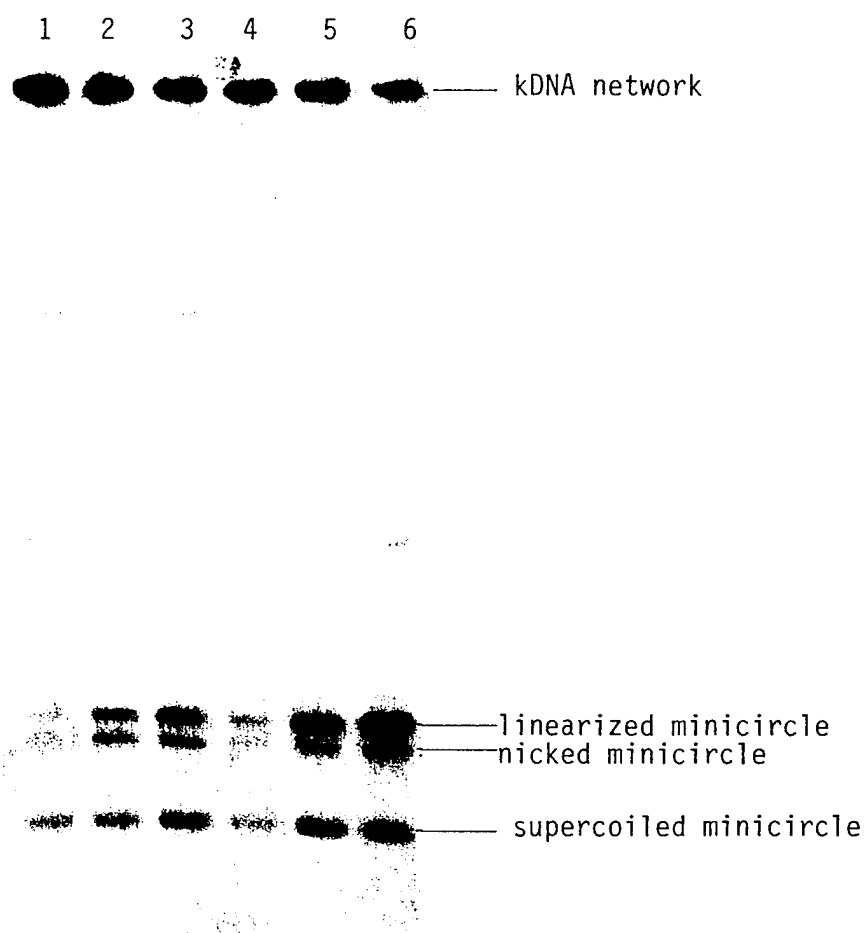


Figure 10. Southern blot of WR242511 treatment for varying times. Lane #1, 2, 3, 4, 5 and 6 correspond to 20 μ M WR242511 for 0, 15, 30 min and 1, 1.5 and 2 hours.

V. DISCUSSION

The results of our investigations demonstrate that the 8-aminoquinoline compounds strongly inhibit the growth of *Leishmania* cells at very low concentrations. At higher concentrations, they are toxic to human cells. WR242511 exhibited the lower IC₅₀ compared with other 8-aminoquinoline compounds.

Our incorporation studies indicate, WR242511 inhibits DNA synthesis initially. DNA synthesis might be the molecular target of 8-aminoquinoline action. Due to a decrease in DNA replication, the rate of RNA synthesis declines. The decreased rate of fatty acid synthesis might be indirectly inhibited due to the decrease of the production of some enzymes involved in fatty acid synthesis. The inhibition of protein synthesis was the least affected metabolic pathway in our investigation.

According to the results from free minicircle DNA assay, WR242511 treatment increased the free minicircle DNA and decreased the catenated minicircle in the kinetoplast network in mitochondria. The concentrations of WR242511, at which the growth of *Leishmania mexicana* 227 was significantly inhibited, corresponds to those concentrations at which the free minicircle DNA in the mitochondria was increased. WR242511 increased the free minicircle and decreased the catenated minicircle DNA within the first 15 min. These results are consistent with our DNA substrate incorporation assay, which demonstrated that DNA synthesis is affected first by WR242511 within the first half hour, then RNA, fatty acid and protein synthesis is inhibited. The results suggest that WR242511 might inhibit the activity

of type II topoisomerase so that the catenation of minicircle to network is inhibited, which results in the inhibition of the parasites growth and division.

Type II topoisomerase can decatenate and catenate the minicircle DNA with the kinetoplast network. The increase of free minicircle might be due to the increase of the decatenation of minicircle or the decrease of catenation of minicircle. The actual mode of action is unknown.

Our next experiments will be to partially purify the type II topoisomerases from leishmanial and malarial parasites, and then test their activity of decatenation, relaxation and catenation with WR242511 *in vitro*.

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RESEARCH ON THE DNA POLYMERASES OF LEISHMANIA MEXICANA

Significance and Justification

There exists a great need to investigate the biochemistry of the mitochondrial DNA polymerase of leishmanial and malarial parasites to elucidate the action and molecular biology of this little studied enzyme and its role in the transformation of this organism into various morphogenic forms and survival in extremely diverse environments.

The information obtained from these investigations will provide (a) insight into the basic biochemistry of this enzyme, (b) the identification of specific inhibitor(s) will provide a useful tool to elucidate the in vivo role of the enzyme and the production of polyclonal antibodies will allow us and others investigators in the field to use it to compare with mitochondrial DNA polymerase from other sources.

The identification of selective inhibitors especially natural will provide potential for the development of novel approaches to the study and function of DNA polymerases in general and increase our understanding of gene function and of parasite proliferation and differentiation. This research will provide insight into basic biochemical mechanisms which could possibly lead to a target for chemotherapeutic exploitation.

Previous Research & Present Outlook

The P.I. has had extensive experience on the propagation of Leishmania sp and culturing and has extensive experience in

the area of isolation and characterization of purine metabolic enzymes. The P.I.'s laboratory has a number of publications on the DNA polymerases α -like and β -like from Leishmania mexicana.

Dr. Naseema Khan will be joining my laboratory in January of 1995. She has had extensive experience on the isolation, characterization and the use of inhibitors to selectively target a specific class of polymerases. Dr. Khan's studies have produced valuable information on the selective identification of various polymerases and the synthesis of novel compounds valuable in the elucidation of DNA replication. She is considered a leader in the polymerase field.

Previously we have focused on the α -like and β -like polymerases of Leishmania mexicana, but the focus of this year's research was on the isolation, characterization and development of an immunochemical assay for the mitochondrial DNA polymerase. The basic science obtained from such studies will elucidate the role and function of the most unique kDNA found in nature.

SUMMARY OF RESEARCH TO DATE

The investigators have isolated two DNA polymerases and partially purified and characterized them from the lower eukaryotic parasitic protozoan Leishmania mexicana. A DNA polymerase α -like (Pol A) was separated from a β -like (Pol B) on a DNA cellulose column. The isolation procedure yielded

an N-ethylmaleimide sensitive peak (A) with an approximate MW of 151,000, and a N-ethylmaleimide resistant peak (B) of MW 40,000. These enzymes were compared to each other and to their mammalian counterpart regarding pH optimum, utilization of various templates, and response to various inhibitors and ionic strengths.

Inhibitor Studies

Exposure of the enzymes to specific DNA polymerase inhibitors showed the L. mexicana enzymes to be different from one another and from mammalian enzymes in their sensitivity to various compounds (Table 1).

Our characterization studies have shown the L. mexicana pol A and pol B to differ from each other in molecular weight, pH optimum, template specificity, and response to salt and inhibitors. In addition, our studies have shown that pol A and pol B share similar properties such as pH optimum, molecular weight, and sensitivity to specific inhibitors such as NEM with their mammalian counterparts.

Observations of differences with the mamalian polymerase(s) α , β and γ have been made on the enzymes of other protozoans (1-8), suggesting that DNA replication in eukaryotes and protozoans may differ. Also, using enzyme neutralization studies, as well as immunodiffusion and immunoelectrophoresis demonstrated that polymerases α and β -like from Leishmania did not crossreact with mammalian polymerase antibodies (1-8,12). Such differences are being

TABLE I: EFFECTS OF BuPdGTP ON SELECTED DNA POLYMERASES AND DNA REPLICATION SYSTEMS
FROM NON-MAMMALIAN SOURCES

| ENZYME OR SYSTEM | LABORATORY OR SOURCE | SUSCEPTIBILITY* | ESTIMATED K_i^1 |
|---|---|--|---|
| <u>E. coli</u> pol I | This laboratory | Resistant | not possible to estimate |
| <u>E. coli</u> pol I, large Klenow fragment | Dr. Samuel Wilson and this laboratory | Resistant | not possible to estimate |
| <u>B. subtilis</u> pol III | This laboratory | Resistant | not possible to estimate |
| <u>Rheo</u> embryo polymerases: pol A pol B pol C I pol C II | Dr. Simon Litvak " " " " | Sensitive Resistant Sensitive Sensitive | < 5 μ M not possible to estimate < 1 μ M > 100 μ M |
| <u>Saccharomyces</u> pol I | Dr. Akio Sugino | Sensitive | 50nM |
| <u>Drosophila</u> pol α | Dr. J. R. Lehman (Dr. Ron Conaway) | Sensitive | < 1 μ M |
| <u>Xenopus</u> pol α | Dr. Simon Litvak | Sensitive | < 1 μ M |
| <u>Epstein Barr Virus (EBV)</u> -Specific polymerase | Dr. Wendy Clough | Sensitive | < 10nM |
| <u>Herpes simplex virus (HSV-1 and HSV-2)</u> -specific polymerases | Dr. Yung-Chi Cheng | Sensitive | 3 μ M |
| <u>Adenovirus</u> -specific replication system (requires Adeno-specific polymerase) | Dr. Thomas Kelly (Dr. Jeff Ostrove) | Sensitive | 50-100 μ M |
| <u>Vaccinia virus</u> specific polymerase | Dr. Samuel Wilson | Sensitive | < 10 μ M |
| <u>Bacteriophage T7</u> -Specific polymerase | Dr. Charles Richardson (Dr. Robert Lechner) | Sensitive | < 10nM |
| <u>Permeabilized Chinese Hamster Embryo Fibroblast</u> system (Ribonucleoside 5'-phosphate dependent) | Dr. Arthur Pardee (Dr. Prem Reddy) | Sensitive | < 1 μ M |

*The term resistant is applied to systems or enzymes which were not inhibited by 500 μ M BuPdGTP in the presence of dGTP at a concentration of 100 μ M or less.

Table (Khan et al, 1984)

characterized in our laboratory. Utilizing the method of Torri & Englund who performed studies on a related kinetoplast of the genus Crithidia, we have demonstrated the existence of the mitochondrial DNA polymerase; have demonstrated associated exonuclease activity and have determined that the enzyme incorporates BuPdGTP and is neutralized by polyclonal antibodies to DNA polymerase from the related protozoan Crithidia fasciculata. Antibodies were obtained Torri & Englund (8).

To summarize, our laboratory has been the first to isolate and characterize the α and β -like DNA polymerases from the lower eukaryotic parasitic protozoan from the genus Leishmania. We are in the process of isolating and characterizing the polymerase γ , which is localized in the mitochondria.

Plan of Work for Coming Year

Growth of Leishmania mexicana for colony maintenance and growth for enzyme purification have been published by the P.I. (under pertinent publications) and the P.I. has had 15 years experience conducting research with this genus. Therefore, details will not be elaborated here. The experiments described below will all be replicated several times to allow statistical analysis if quantitative data are collected.

**TO PURIFY MITOCHONDRIAL DNA POLYMERASE FROM LEISHMANIA
MEXICANA PROMASTIGOTES.**

Promastigotes can be grown easily and in sufficient quantity (100L) to provide the necessary protein and we will use the methods of Torri and Englund (8) who isolated this enzyme from a related Kinetoplast, Crithidia fasciculate. These investigators modified their procedure from that of Wernette & Kaguni (13). In contrast to these investigators, we will use the method of Holmquist (22) to isolate intact mitochondria by a hybrid Percoll/metrizamide discontinuous density gradient from the postnuclear supernatant. Centrifugation is for a minimum of 15 minutes at 50,000 g (20,000 rpm) at 4°C. Mitochondria band at the 17/35% metrizamide interface.

The mitochondria will be lysed as described (Torri, 9) and the polymerase will be isolated utilizing hydroxylapatite chromatography, single-standard DNA cellulose chromatography, mono S FPLC, and sedimentation on a glycerol gradient. After each step, fractions will be assayed for activity and purity by SDS-PAGE analysis (Laemmili) and coomassie-blue staining (15).

The DNA polymerase assay will be that of Torri & Englund (8) and protease inhibitors will be present to prevent proteolysis.

DNA Polymerase Assay

The reactions (50 ul) will contain 50 mM Tris-HCl (pH 9.

0), 5 mM MgCl_2 , 0.1 mg/ml BSA, 30 pM [α - ^{32}P]dTTP (1000-3000 Ci/mmol), and 0.15 mg/ml activated calf thymus DNA. Only a single deoxynucleoside triphosphate is used in this assay (8). Similar to the results of Torri & Englund, we have found that the presence of all four dNTP in the assay mix decreases the incorporation of labeled nucleotide. After 15 min at 37°C, the DNA will be precipitated by the addition of 900 ul of 2 M HClO_4 , 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$. The samples will be collected under vacuum on GF/C filters (Whatman) and washed.

Polymerization in situ (16,17) will be used to determine which polypeptides are associated with the near-homogenous enzyme. The crude and highly purified enzymes will be subjected to activity gel analysis. The procedure involves denaturation and electrophoresis of the enzyme in a DNA-polyacrylamide gel followed by renaturation and enzyme assay in situ. Purity will be assessed at all stages with analytical PAGE. If not homogenous, then further gel or affinity chromatography will be tried.

CHARACTERIZATION OF THE MITOCHONDRIAL POLYMERASE

Once sufficient enzyme is available then characterization and further purification of the polymerase to near homogeneity will proceed. Initially the activity in partially purified leishmanial mitochondria enzyme will be analyzed for properties such as optimal pH, salt, metal ion requirement, template and natural template-primers -- such as, Activated calf thymus DNA, kDNA from the leishmanial parasite

(to be isolated by the method of polydA-oligo dT, poly rA-oligo dT, poly dC-oligo dG, poly dT-oligo 1A, poly dC, poly dG, etc. These assays will be performed varying the concentration of Mg^{2+} and Mn^{2+} .

The use of classical DNA polymerase inhibitors will be tested to further characterize and distinguish polymerase activities in leishmanial preparations. Inhibitors to be tested include: aphidicolin, N-ethyl maleimide, butylphenyl-dGTP, dideoxynucleoside 5'-triphosphates, and carbonyldiphosphonate and natural plant products and fatty acids. In general mitochondrial DNA polymerase is aphidicolin resistant but N-ethylmaleimide sensitive and is moderately inhibited by dideoxythymidine triphosphate (9). Their observation is consistent with our preliminary data using partially purified enzyme. Other compounds which will be tested include antitrypanosomals such as sinefungin, berenil which have been shown to inhibit the synthesis of kDNA in vivo in leishmanial cells in the P.I.'s laboratory, and other antileishmanials such as pentavalent antimony compounds (sodium stibogluconate and N-methyl-glucamine antimonate).

Dr. Naseema Khan has been a member of the research team of Drs. Neal Brown and George Wright (University of Massachusetts Medical Center) for the past 15 years and is expert in the analysis of DNA polymerases with the use of selective inhibitors which have been designed, synthesized and tested by their respective laboratories and

collaborations. Their joint research efforts with the use and synthesis of selective inhibitors of DNA polymerases have been of extreme importance in classification of the polymerase enzymes from different sources and as probes of the function of polymerases in intact cells. The goals of their research efforts have been the design of selective agents to explore the structures of polymerases, to assist in the identification of in vivo polymerase function, and in the development of cytotoxic agents.

Kinetics and inhibition analysis will be determined for inhibitors at various stages of enzyme purification. Competitive inhibitors will also be tested in a truncated assay which lacks the competitor dNTP (10,16,17). PAGE analysis of incorporation of analogs will be performed. The products will be separated on a 12% polyacrylamide gel by electrophoresis at 1500 volts for 4h, and the dried gel will be exposed to X-ray film as described (10,16,17,21).

One of our objectives to purify DNA polymerase γ is to further explore the active site of DNA polymerases (Pol A, Pol B) from Leishmania mexicana. We will compare the mechanism of inhibition and substrate potential of BuPdGTP by DNA Pol A, Pol B and Pol γ from Leishmania. BuPdGTP inhibits eukaryotic DNA polymerase with high potency IC_{50} 0.026 μ M, but is not a substrate for the enzyme (24) (Khan et al, 1991). This lack of ability to incorporate BuPd is also not shared by another sensitive DNA polymerase that from the bacteriophage T4. The

T4 enzyme is inhibited with nanomolar potency by BuPdGTP, but is able to incorporate the analog (25) (Khan et al, 1991,1994).

E. coli DNA polymerase I (Klenow) has been considered resistant to BuPdGTP, based on weak inhibition (26) (Misra et al, 1992) or lack of inhibition (27) (Khan et al, 1984) under standard assay conditions of enzyme in the presence of activated DNA. BuPdGTP surprisingly has good affinity for *E. coli* DNA polymerase I.

BuPdGTP shows intermediate sensitivity to 29 DNA polymerase (Blasco et al, 1992) and also acts as a substrate for 29 DNA polymerase. HSV₁ polymerase also shows moderate inhibition and also incorporates the inhibitor (Khan et al, 1994).

In our preliminary study by using partially purified DNA polymerase from Leishmania mexicana we found that DNA pol α is moderately inhibited by BuPdGTP and the inhibitor at 10 μ M concentration acts as a substrate for DNA polymerase γ .

This is a very interesting observation because the information will help us and other investigators to better understand the mechanism of inhibition of DNA polymerase(s) in general since most of the work to elucidate the active site of DNA polymerase is done on Pol α type of enzymes.

Since the P.I. is a member of the International Biodiversity Group involving Walter Reed Institute of Research and various universities in Nigeria and Cameron, and the

National Cancer Institute, access to medicinal plant (from the Rain Forest) extracts and isolated products will be available to test. Collection of these materials are being based on ethanobotanical and epidemiological investigations of indigenous populations. The P.I. has personally collected medicinal plants from the Huaorani tribe from the Amazon Basin in Ecuador and is presently conducting investigations on the effect of natural products on DNA replication. Since the leishmanial parasite spends part of its life cycle in an insect vector, it will be of interest to determine how plant products which serve as the vector's nutritional source affects DNA polymerase function and gene expression in the polymorphic leishmanial parasite. Recently a number of publications have described the isolation of active agents from plants used by indigenous people for the treatment of leishmaniasis. The compounds isolated (naphthoquinones, plumbagin, triterpene and lupeol) will first be tested in vitro with H^3 thymidine or P^{32} phosphate to determine if DNA synthesis is inhibited. If compounds are found to be inhibitory, they will be tested with isolated enzyme fractions. Also, the effect of the naturally occurring polyamines, spermidine and spermine, as well as the diamine putrescine and arachidonic acid (previously discussed) will be studied.

Recently Gonzalez et al (14) has shown that "in vitro" growth of Leishmania mexicana promastigotes can be fully

controlled by the intracellular levels of polyamines which can be regulated by inhibition of their biosynthesis and limitation of their supply from the external medium.

Our laboratory (unpublished data) and others working either with Leishmania or other lower eukaryotic parasitic protozoa have observed that synthesis of DNA is catalyzed by a polyamine dependent α -like DNA polymerase. We would like to further characterize the effect of polyamines on DNA synthesis by observing their effect on mitochondrial DNA polymerase.

Protein structure and enzymatic properties for DNA polymerase from mitochondria (gamma) has been controversial due to its low abundance and proteolytic degradation during purification. The P.I. has been isolating various enzymes from the DNA synthetic pathway including DNA polymerases from Leishmania sp for the past 15 years and is acutely aware of the strong proteolytic activities present in these organisms making enzyme purification and stability difficult. Dr. Khan has been isolating various polymerases from a variety of sources for the past 15 years and is expert in the use of proteolytic inhibitors and stabilizers to maintain native protein structure. Our publications describe the use of inhibitors and stabilization techniques (see pertinent publication list of P.I. and Co-P.I.).

Physical Properties

Physical properties such as homogeneity, sedimentation coefficient, molecular mass and subunit structure will be

determined.

Molecular Mass

The native molecular mass of the mitochondrial DNA polymerase will be estimated by combining the sedimentation coefficient with the Stokes radius as determined by Sephacryl S-200 gel filtration in the presence of 0.2 M $(\text{NH}_4)_2\text{SO}_4$. To monitor copurification with other proteins during purification the following enzymes will be tested for coelution to determine if perhaps they form a complex associated with polymerase activity. DNA dependent ATPase to be assayed by the Focher et al (18), 3-5' exonuclease activity to be determined by the method of Byrnes et al (11) and topoisomerases (19). Assays will be performed during different stages of purification to determine if calf thymus proliferating cell nuclear antigen (PCNA) can stimulate the DNA polymerase (18).

Subunit structure will be determined by SDS-polyacrylamide gel electrophoresis of peak fractions during the last stage of purification as described by Wernette and Kaguni (13). To determine if the polypeptides associated with the enzyme exhibit DNA polymerase activity, the crude enzyme and highly purified enzymes will be subjected to activity gel analysis by the method of Hubscher (20).

Results will be compared to the other four classes of DNA polymerases and to mitochondrial polymerases reported in the literature.

**TO RAISE POLYCLONAL ANTIBODIES AND DEVELOP AN IMMUNOASSAY FOR
LEISHMANIAL MITOCHONDRIAL DNA POLYMERASE.**

Once enough pure DNA polymerase is obtained, polyclonal antibodies will be raised in rabbits and immunoassays developed. Dr. Khan's laboratory has had considerable experience raising antibodies and developing immunoassays (21). The animal work will be performed by Dr. Ronald Labbe who routinely keeps rabbits and has animal facilities available and has permission to perform animal studies through our animal care office. In the case that there is not enough purified enzyme to serve as both an antigen and as standard for making of the standard curve in an immunoassay, we will take advantage of the partial amino acid sequence which can be done in our Biotechnology Core facility which has a technician dedicated to the facility. A 25-amino acid synthetic fragment (25-mer) of the polymerase can be produced from the amino acid sequence of our enzyme in the Core Facility according to the identified sequence. This 25-mer can be covalently conjugated to a carrier protein to serve as antigen following conventional procedure of Harlow and Lane (23). Antiserum raised against this 25-mer should also recognize the polymerase itself through its section corresponding to the 25-mer. The synthetic 25-mer will also serve as an abundant source of standard in the immunoassay. Native mitochondrial polymerase will be quantified as 25-mer equivalents.

The P.I.'s lab has a Molecular Device Thermomax

microplate reader for this project.

The immunoassay will be used to detect, quantitate and follow the production of mitochondrial DNA polymerase in the growth of this lower eukaryotic protozoan kinetoplast and in the presence of various natural and synthetic polymerase inhibitors. Basic information of the role this enzyme plays in the life cycle of this polymorphic organism as well as the effect antileishmanial and DNA polymerase inhibitors have on the synthesis and function of the polymerase can be expedited by this immunoassay.

The availability of this assay will also facilitate the research of other investigators in the field who wish to compare mitochondrial polymerases from other kinetoplasts as well as other prokaryotic and eukaryotic organisms.

Publications Resulting from Current Contract (all or in part)

Refereed and Published

1. Nolan, L., Tang, S. and Sufrin, J., Partial Purification and Characterization of the Isozymes of S-Adenosylmethionine Synthetase from Leishmania mexicana, American Society of Microbiology 1, 1994, pg. 67.
2. Swenson, J., Nolan, L., McClure, C., Antileishmanial Properties of Herbs and Amazonian Plants, American Society of Microbiology 1, 1994, pp. 19.
3. McClure, C., Nolan, L., Zatycka, S., Ponce del Leon, F. Effect of Medicinal Plant Extracts on the Growth of Leishmania sp. and Mammalian Cell Lines. American Society of Microbiology 1, 1994, pg. 18.
4. Liu, H. and Nolan, L.L. The Mode of Action of 8-Aminoquinolines and Their Synergy with Antileishmanial Compounds on Leishmania mexicana. American Society of Microbiology 1, 1994, pp. 17.
5. McClure, C., Nolan, L.L. and Swenson, J. Effect of Allium sp. and other Natural Herb Extracts on the Growth of Leishmania sp. and Mammalian Cell Lines. American Society for Microbiology 1(11), 1993, pg. 7.
6. Liu, H. and Nolan, L. The Effects of 8-Aminoquinoline on Leishmania and Human Cells. American Society of Microbiology 1(11) 1993, p. 8.
7. Nolan, L.L., Rivera, J. and Zatycka, S. Inhibition of DNA Polymerase Activity by Arachidonic Acid in Leishmania mexicana and Prokaryotic and Eukaryotic Sources. American Society of Microbiology 1(11), pg. 10.
8. Nolan, L. S-Adenosylmethionine Synthetase from Leishmania mexicana. American Society of Microbiology-Biotechnology, 1993, pg. 4.
9. Nolan, L.L. and Labbe, R.G. Effect of Allium sp. and herb extracts on Leishmania, mammalian cells and food-borne pathogens, International Union of Microbiology Proceedings, 1, 1994, pg. 94.
10. Nolan, L.L. and Rivera, J. Mode of action of arachidonic acid in Leishmania mexicana, American Society of Parasitology, Proceedings, 1994, pg. 22-25.

1. Presented - American Society of Microbiology, Las Vegas, Nevada, 5/94.
2. Presented - American Society of Microbiology, Las Vegas, Nevada, 5/94.
3. Presented -National Undergraduate Research, Kalamazoo, MI, 3/94.
4. Presented - American Society of Microbiology, Boston, MA, 11/93.
5. Presented - American Society of Microbiology, Boston, MA, 11/93.
6. Presented - American Society of Microbiology, Boston, MA, 11/93.
7. Presented - American Society of Microbiology, Boston, MA, 11/93.
8. Presented - American Society of Microbiology, Boston, MA, 11/93.
9. Presented - Prague, Czech Republic, July 1994.
10. Presented - Ft. Collins, CO, 8/94.